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**THE BIODEGRADATION OF
HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE
(RDX)**

By

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May 1981

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Biodegradation of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) occurs under anaerobic conditions to yield a number of products including: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, hexahydro-1,3,5-trinitroso-1,3,5-triazine, hydrazine, 1,1-dimethylhydrazine, 1,2-dimethylhydrazine, formaldehyde, and methanol. A scheme for the biodegradation is proposed which proceeds via successive reduction of the nitro groups to a point where destabilization and fragmentation of the ring occurs. The non-cyclic degradation products arise via subsequent reduction and		

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rearrangement reactions of the fragments.

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PREFACE

Manufacturing and loading operations involving military munitions such as RDX and HMX cause some environmental pollution at the sites of these activities. The purpose of the present effort was to determine the biodegradation products and/or the ultimate degradability of these materials in the environment. The work was conducted at the US Army Natick Laboratories, Natick, MA, and was funded by the Chemical Systems Laboratory, US Army ARRADCOM, Aberdeen Proving Ground, MD, as work unit W-20 under Project 1L762720D048 (Environmental Quality, R&D).

If these efforts indicate that a particular scheme is effective in degrading these components, then the process may be scaled up to full operation, and utilized at RDX/HMX manufacturing and loading sites, in order to bring the waste effluents to EPA discharge standards.

The authors thank Carmine Di Pietro for performing the mass spectral analyses, and acknowledge the assistance of Diana Foster, Deborah Rollins, Michael Whalen, Linda Faulk and Barry Jacobs in certain phases of this work. We wish to express appreciation to John Hoffsommer for his helpful discussions.

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THE BIODEGRADATION OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX)

INTRODUCTION

Among pollutants unique to the military are those arising from the manufacture, handling and demilitarization of munitions. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is an explosive widely used for military purpose. A homolog octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) is a by-product of the synthesis of RDX and is also used in RDX formulations. During the manufacture of RDX, up to 12 µg/ml may be discharged to the environment in process waste waters.¹

The question of toxicity of RDX to aquatic life has been addressed^{2,3} and studies conducted by the Office of The Surgeon General have recommended a 24-hour average maximum allowable concentration of 0.30 µg of RDX/ml of waste water to protect aquatic life.³ It has not been clearly established whether or not RDX is toxic in mammalian toxicity studied.^{4,5,6} These reports suggest that RDX per se may not present a serious toxicity problem but that metabolic products derived from RDX may be toxic.

¹Jackson, R. A., et al. 1978. Nitramine (RDX/HMX) wastewater treatment at the Holston Army Ammunition Plant. Technical Report ARLCD-CR-77013. U.S. Armament Army Res. and Devel. Com., Dover, NJ.

²Glennon, J. P., and L. H. Reuter. 1976. Environmental quality standards for munitions-unique pollutants. Proc. 17th Dept. of Army Explosives Safety Seminar, Denver, CO.

³Sullivan, J. H., et al. 1979. A summary and evaluation of aquatic environmental data in relation to establishing water quality criteria for munitions unique compounds. Part 4: RDX and HMX. Technical Report DAMD-17-77-C-7027, U.S. Army Med. Res. and Devel. Com., Ft. Detrick, Frederick, MD.

⁴Hathaway, J. A., and C. R. Buck. 1977. Absence of health hazards associated with RDX manufacture and use. J. Occup. Med. 19:269-272.

⁵Ross, E. R. 1976. Two-year chronic toxicity study in rats. U.S. NTIS, AD Rep. AD-A040161.

⁶Schneider, N. R., et al. 1977. Toxicology of cyclotrimethylenetrinitramine (RDX): distribution and metabolism in the rat and the miniature swine. Toxicol. Appl. Pharmacol. 39:531-541.

A number of studies on the products formed from the chemical decomposition of RDX have been reported. Hoffsommer et al.⁷ reported that the alkaline hydrolysis of RDX yielded nitrate, nitrogen, ammonia, nitrous oxide, formic acid, formaldehyde, and traces of H₂. The first step in this reaction was a proton abstraction by the base with simultaneous elimination of a nitrate group from the adjacent ring nitrogen.⁸ Exposure of aqueous solutions of RDX to ultra-violet light (UV) resulted in the formation of nitrate, nitrite, ammonia, formaldehyde, nitrous oxide, formamide, and N-nitroso-methylenediamine.⁹ The combination of UV with ozone produced CO₂, cyanic acid, nitrate, ammonia and formic acid.¹⁰

The decomposition of RDX in biological system has been reported. Osmon and Klausmeier¹¹ found that some RDX disappeared during soil enrichment studies, but evidence for RDX degradation by microorganisms was not obtained. The complete disappearance of RDX by mixed cultures of purple photosynthetic bacteria was reported by Soli,¹² who advanced the hypothesis that the strongly reducing conditions of the culture during photosynthesis were responsible for

⁷Hoffsommer, J. C., et al. 1977. Kinetic isotope effects and intermediate formation for the aqueous alkaline homogeneous hydrolysis of 1,3,5-triaza-1,3,5-trinitrocyclohexane (RDX). J. Phys. Chem. 81:380-385.

⁸Croce, M., and Y. Okamoto. 1978. Cationic micellar catalysis of the aqueous alkaline hydrolysis of 1,3,5-triaza-1,3,5-trinitrocyclohexane and 1,3,5,7-tetraaza-1,3,5,7-tetranitrocyclooctane. J. Org. Chem. 44:2100-2103.

⁹Glover, D. J. and J. C. Hoffsommer. 1979a. Photolysis of RDX. Identification and reactions of products. Technical Report NSWC TR-79-349. Naval Surf. Weap. Center, Silver Springs, MD.

¹⁰Glover, D. J. and J. C. Hoffsommer. 1979b. Photolysis of RDX in aqueous solution, with and without ozone. NSWC/WOL TR-78-175. Naval Surf. Weap. Center, Silver Springs, MD.

¹¹Osmon, J. and R. E. Klausmeier. 1973. Microbial degradation of explosives. Dev. Ind. Microbiol. 14:247-252.

¹²Soli, G. 1973. Microbial degradation of cyclonite (RDX). U.S. Natl. Tech. Inform. Serv., AD Report No. 762751.

disruption of the RDX molecule. Hoffsommer et al.¹³ found no disappearance of RDX in an aerobic activated sludge system. Sikka¹⁴ reported the disappearance of RDX, after a 20-day lag period, from river water samples supplemented with river sediment. The observation was made that $^{14}\text{CO}_2$ was evolved from ^{14}C -RDX depending on the source of the sediment.

The products of the biological degradation of RDX may pose more serious toxological problems than the RDX itself. Therefore, it is important not only to assess the environmental fate of RDX, but also to determine the nature of the products of biotransformation and/or biodegradation.

¹³Hoffsommer, J. C., et al. 1978. Biodegradability of TNT: A three year pilot study. NSW/C/WOL TR-77-136.

¹⁴Sikka, H. C., et al. 1980. Environmental fate of RDX and TNT. Technical Report DAMD 17-77-C-7026.

METHODS

Cultures and Media Biodegradation studies were carried out either in nutrient broth (Difco) or in a medium consisting of 0.04 M KNO_3 and 0.005 M K_2HPO_4 , supplemented with 0.3% (v/v) of beet-sugar molasses. For aerobic studies the cultures were inoculated with activated sludge obtained from the Marlboro Easterly Municipal Sewage Treatment Plant, Marlboro, MA. The volume of liquid did not exceed 10% of the total volume of the flask; the flasks were incubated at 30°C on a reciprocating shaker (150 strokes/min). Anaerobic cultures were inoculated with anaerobic sewage sludge obtained from the Nut Island Sewage Treatment Plant, Boston, MA. For anaerobic studies the vessels were filled to approximately 95% of their capacity, inoculated and incubated as stationary cultures at 37°C. The inocula were prepared by diluting the sludge with 2 volumes of distilled water and filtering through glass wool. A 2% (v/v) inoculum was used.

Due to the relative insolubilities of the compounds used as substrates, weighed amounts were added to empty flasks and dissolved in a small amount of acetone. The acetone was evaporated by a stream of N_2 , leaving a thin film of material on the inside surface of the flask. The culture medium was deoxygenated by boiling, poured into the vessel, and the mixture stirred vigorously until solution was attained. The medium was allowed to cool to 35-40°C before inoculation. Samples were removed from the culture vessels after various periods of incubation, centrifuged, and the supernatant solutions filtered through 0.22- μm membrane filters. The resulting culture medium filtrates (CMF) were used for all analytical procedures.

Liquid Chromatography Samples of CMF (10 to 20 μl) were injected without further treatment into a Waters Model 2000A Liquid Chromatograph equipped with a

μ Bondapak C-18 column (Waters Associates, Milford, MA). The solvent system used to monitor the disappearance of RDX was 20% methanol in water; solvent flow was 2.5 to 3.0 ml/min; UV detector at 230 nm. For determination of formaldehyde, the solvent was 60% methanol in water; UV detector at 340 nm.

Vacuum Distillation Samples of CMF (100 ml) were placed in a 500-ml boiling flask fitted with an adaptor to which an empty flask was attached. The CMF was frozen in liquid N_2 , a vacuum (0.01 mm Hg, 1.3 Pa) was established, and the system was closed off. The empty flask was placed in liquid N_2 and the sample-containing flask was allowed to warm. After 2 hours the system was opened to atmospheric pressure, and the colorless material which had collected in the second flask was assayed for radioactivity and subjected to GC/MS analysis for the presence of methanol.

Determination of Formaldehyde CMF (25 ml) was adjusted to pH 2 with H_2SO_4 and distilled. The distillate was collected with the delivery tube submerged below the surface of a small amount of H_2O in order to retain volatile components. To 100 ml of distillate was added 1 ml of 10% 5,5-dimethyl-1,3-cyclohexanedione (methone) in 95% ethanol. The mixture was heated to boiling for 5 min, cooled and stored at 4°C for several days to allow crystallization to occur. Crystals were collected, washed with a small amount of ice-cold H_2O and dried; m.p. 192°C; literature (lit.) 189°C.¹⁵ The colorimetric determination of formaldehyde was carried out on 0.1 to 1.0 ml portions of distillate using the chromotropic acid method as described by Grant.¹⁶ The absorbance was determined at 570 nm with a

¹⁵Walker, J. F. 1944. Formaldehyde. Reinhold Publishing Corp., New York, NY.

¹⁶Grant, W. M. 1948. Colorimetric microdetermination of formic acid based on reduction to formaldehyde. Anal. Chem. 20:267-269.

Coleman Jr. Spectrophotometer. An HPLC method for determining formaldehyde directly in aqueous systems based on a method described by Beasley et al.¹⁷ for the determination of formaldehyde in air was developed. The method involved the addition of one volume of a filtered 0.1% solution of 2,4-dinitrophenylhydrazine in 2 N HCl to an equal volume of CMF contained in a small test tube. The contents were mixed, allowed to react for 10 min. and 10-20 μ l were injected directly into the HPLC. A reference standard was prepared from recrystallized formyl-2,4-dinitrophenylhydrazone; m.p. 166°C; lit. 166°C.¹⁸ Unreacted reagent eluted at 2.45 min. and the formyl derivative at 4.36 min. Figure 1 shows a standard curve prepared from 10^{-4} diln of 37% formaldehyde and illustrates the linearity achieved and the sensitivity of the assay. The assay is not affected by large concentrations of acetaldehyde or by acetone, ethanol, or formate as shown in Table 1.

Synthesis of ^{14}C -RDX The synthesis of ^{14}C -RDX was based on the method described by Schiessler and Ross.¹⁹ The reaction was conducted in the screw-capped vial (60 mm x 17 mm) in which ^{14}C -paraformaldehyde (1 mCi, 2.3 mg) was received (New England Nuclear). The cap was fitted with a Teflon liner and 23.4 mg of finely powdered paraformaldehyde was added to the vial to bring the total paraformaldehyde concn. to 0.85 mmol. Finely powdered NH_4NO_3 (68.5 mg. 0.85 mmol) was introduced into the vial, together with a 10-mm Teflon-clad miniature stirring bar. Acetic anhydride (0.3 ml, 3.2 mmol) was added, followed by 50 μ l of boron trifluoride etherate. The vial was tightly closed and heated for 7 hours at 65-70°C with

¹⁷Beasley, R. K., et al. 1980. Sampling of formaldehyde in air with coated solid sorbent and determination by high performance liquid chromatography. Anal. Chem. 52:1110-1114.

¹⁸Shriner, R. L. and R. C. Fuson. 1948. The systematic identification of organic compounds. John Wiley and Sons, Inc., New York, NY.

¹⁹Schiessler, R. W. and J. H. Ross. 1948. U.S. Patent 2,434,230.

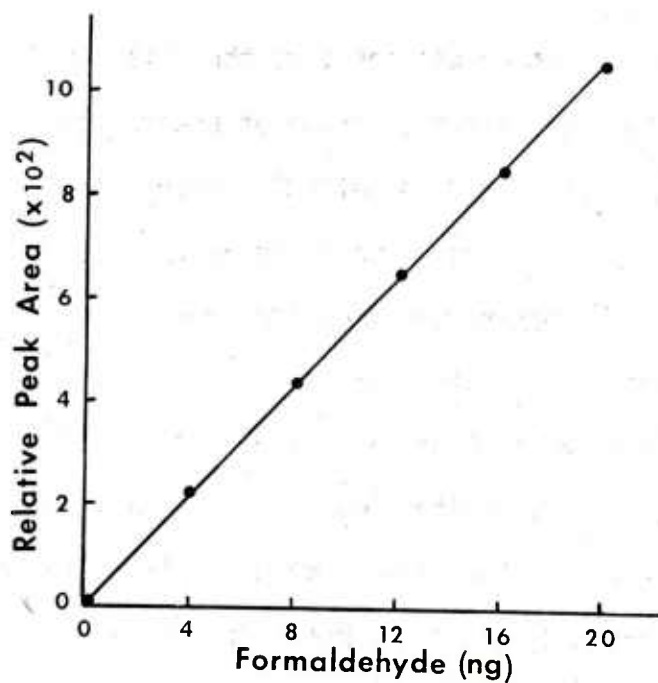


Figure 1. HPLC assay for formaldehyde.

Table 1. Effect of other compounds on the formaldehyde assay.

Additions	Relative Peak Area at 4.36 min	
	no HCHO	+ 10 ng HCHO
None		518
Nutr. Broth (2X) ^a	0	514
CH ₃ CHO (100X) ^b	0	525
Acetone (10 ⁶ X) ^b	0	525
Ethanol (10 ⁶ X) ^b	0	517
Formate (100X) ^b	0	511

^a Double strength nutrient broth was used.

^b Numbers in parentheses indicate the fold excess over the concn. of HCHO used.

stirring. At the end of the reaction time the vial was opened and the stirrer was removed and washed with several drops of acetic acid. The product was precipitated by the addition of 0.3 ml of H₂O and brought into solution by boiling the reaction mixture. The product was allowed to crystallize at room temperature for several hours. The supernatant solution was removed by decantation and the crystals washed twice with their volume of H₂O. The product was dried under a current of air at 70°C and finally at 0.1 mm (13.3 Pa) Hg at 25°C. The yield of product was 28.9 mg (45.9% of theoretical). The IR spectrum was identical to that of a twice recrystallized authentic sample of RDX obtained from Holston Army Ammunition Plant, Holston, TN. Both samples exhibited the same R_f on cochromatography on silica gel TLC. A trace of HMX was eliminated by recrystallization from acetone.

Synthesis of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) This compound was prepared by the method of Brockman *et al.*²⁰ The crude produce was recrystallized twice from 95% ethanol and then from benzene to give 4.8 g of yellow needles, m.p. 105-107°C; lit. 105-107°C. The recrystallized product gave a single spot when subjected to TLC analysis on Eastman silica gel plates with fluorescent indicator. The developing solvent was benzene/ethanol (95:5) and the material was visualized by fluorescence quenching under UV. The IR spectrum was consistent with the structure, exhibiting bands at 1449 cm⁻¹ and 1495 cm⁻¹ (N-NO). The mass spectrum of the product had a peak at m/e 174, corresponding to the molecular ion, and a major fragment ion at m/e 100.

Synthesis of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) These compounds were synthesized

²⁰Brockman, F. J., *et al.* 1949. Nitrolysis of hexamethylenetetramine. III. Preparation of pure cyclonite. *Canad. J. Res.* 27B:469-474.

from TNX by the method described by Simacek.²¹ The synthesis yielded a mixture of MNX, DNX and unreacted TNX. MNX and DNX were isolated as a single slow moving band by preparative TLC and further purified by HPLC. MNX was subjected to analysis by GC/MS and exhibited a molecular ion at m/e 206 and a major fragment ion at m/e 132. GC/MS analysis of DNX showed a molecular ion at m/e 190 and fragment ion at m/e 116.

Isolation of Hydrazine and its Dimethyl Derivatives CMF was made alkaline with NaOH and distilled in a rotary evaporator at 50°C under 15 mm Hg (2.0 kPa). The distillate was collected in dilute HCl. The colorless distillate was evaporated to dryness in the same manner to yield residue A. For the isolation of salicylazine, residue A was dissolved in a small volume of H₂O and neutralized with NaOH. The resulting solution was shaken with 25 ml of 4% salicylaldehyde in benzene. The benzene layer was separated and evaporated to dryness at room temperature under a stream of N₂. The residue was stored over silica gel in a vacuum desiccator for several days to remove excess salicylaldehyde. The residue was subjected to TLC analysis using benzene as solvent. A reference sample of salicylazine, prepared by the method of Blout and Gofstein,²² was cochromatographed with the unknown. On visualization with UV the TLC exhibited two principal spots, the lower of which had the same R_f as salicylazine. The material was analyzed by GC/MS and two principal peaks were observed. The larger had the same retention time as salicylazine (MW 240) exhibiting a molecular ion at m/e 240. Its spectrum was identical to that of the reference standard. A small peak with molecular ion at m/e 239 was not further identified. For the isolation

²¹Simacek, J. 1957. Decomposition of nitrosamines and nitramines in protogenic solvents. III. Synthesis of N,N'-dinitro-N"-nitrosocyclotrimethylenetriamine. Chem. listy 51:2367-2368.

²²Blout, E. R. and R. M. Gofstein. 1945. The absorption spectra of certain aldazines. J. Am. Chem. Soc. 67:13-17.

of the dimethylhydrazines residue A was refluxed with 10 ml of methanol and cooled to room temperature. The supernatant was removed to a small vial and evaporated to dryness in a block heated at 70°C. The methanol extraction of residue A was repeated twice more with 3 ml each of methanol. The residue from the final evaporation was treated with 250 µl of pyridine and 25 µl of acetic anhydride. The vial was sealed with a Teflon-lined cap and heated at 65°C for 10 min. Standards were concurrently prepared with 3 µl each of methylhydrazine, 1,1-dimethylhydrazine and 1,2-dimethylhydrazine treated in the same manner. An additional standard was prepared containing 3 µl of 1,1-dimethylhydrazine and 10 mg of ammonium chloride in addition to the acetylating mixture. The samples were analyzed using a Bendix Model 2500 gas chromatograph with a 6 ft. (183 cm) Pyrex column (0.25 in., 0.64 cm diam.) packed with Tenax GC. The carrier gas was N₂ at 40 ml/min.; a flame ionization detector was used at 250°C and the injection port was maintained at the same temperature. With the oven temperature at 230°C the derivatized extracts from the CMF gave two small peaks. The peak at 4.6 min. corresponded to acetyl-1,2-dimethylhydrazine; the other peak at 3.5 min. was not identified. No peaks corresponding to acetyl-1,1-dimethylhydrazine (1.6 min.) or acetylmethylhydrazine (4.3 min.) were observed. However, when the oven temperature was reduced to 190°C a larger peak was observed at 4.1 min. corresponding to acetamide-dimethylhydrazone. This compound is formed from 1,1-dimethylhydrazine under derivatization conditions when an excess of ammonium chloride is present, as was the case in CMF. The identities of acetamide-dimethylhydrazone (m/e 101) and acetyl-1,2-dimethylhydrazine (m/e 102) were confirmed by GC/MS.

Distribution of Radioactivity CMF was adjusted to pH 3 with HCl and sparged with helium for 2 hrs. (Figure 2). The gas was passed sequentially through a series of traps which contained 0.1 M Na₂SO₃, 0.1 N HCl, H₂O and 0.1 N NaOH,

respectively. The sparging gas continued through a copper oxide-packed column heated to 700°C, and finally through a second 0.1 N NaOH trap. After purging the acidified mixture for 2 hrs., the mixture was adjusted to pH 11 and the sparging was continued for an additional 2 hrs. The resulting purged aqueous phase was neutralized and subjected to continuous ether extraction for 24 hrs. The aqueous phase was adjusted to pH 3 and similarly extracted with ether. Finally the aqueous phase from the pH 3 ether extraction was adjusted to pH 11 and again extracted with ether for 24 hrs. Samples (usually 1.0 ml) were placed in scintillation vials to which 10 ml of Aquasol-2 (New England Nuclear) were added. Measurements for radioactivity were carried out in a Model 3255 Packard Tri-Carb Liquid Scintillation Spectrometer.

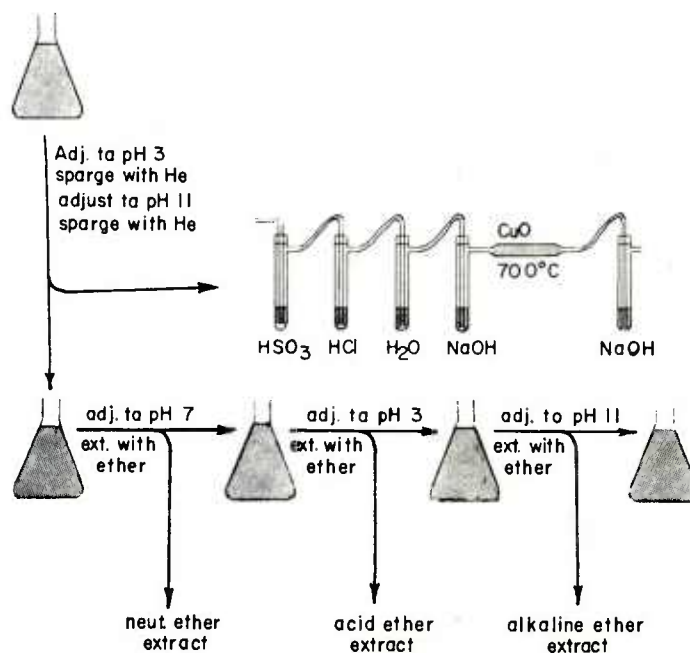


Figure 2. Scheme for examination of reaction mixtures for ^{14}C distribution.

RESULTS

RDX Disappearance

RDX at concentrations of 50 or 100 $\mu\text{g/ml}$ disappeared rapidly from nutrient broth cultures inoculated with anaerobic sewage sludge and incubated anaerobically (Figure 3). RDX disappearance was essentially complete after four days. Concentrations of RDX remained unchanged when cultures were inoculated with aerobic activated sewage sludge and incubated aerobically. No RDX disappeared in uninoculated controls.

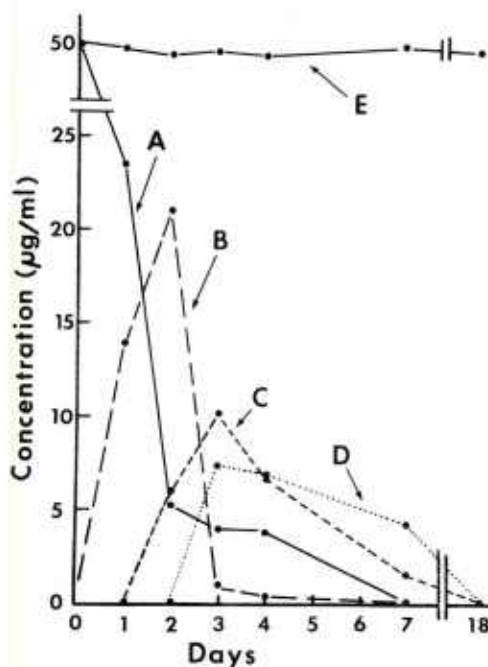


Figure 3. Disappearance of RDX and production of intermediates during anaerobic incubation. A = RDX; B = 1-nitroso-3,5-dinitrohexahydro-1,3,5-triazine; C = 1,3-dinitroso-5-nitro-hexahydro-1,3,5-triazine; D = 1,3,5-trinitroso-hexahydro-1,3,5-triazine; E = RDX incubated under aerobic conditions.

Intermediate Formation

HPLC analysis of anaerobic reaction mixtures revealed the presence of intermediates formed during the disappearance of RDX. Figure 4a shows the HPLC elution pattern at $t=0$. The small amount of HMX present in the RDX is apparent. Figure 4b is the elution pattern obtained at $t=3$ days. The presence of intermediates is readily seen. The kinetics of RDX disappearance and intermediate formation is shown in Figure 3. The fact that the reaction proceeded only under anaerobic conditions suggested that these intermediates might be reduced forms of RDX. Chloroform extraction of an 8-day incubation mixture which initially contained 50 mg of RDX/ml yielded yellow crystals having the same m.p., IR spectrum, and GC/MS as authentic TNX. Extraction of 2-3 day old cultures (appreciable quantities of MNX and DNX present) with ethyl acetate and subsequent 500-fold concn. allowed MNX and DNX to be separated in sufficient quantity by HPLC to establish their identity by GC/MS. As with the synthesized reference compounds a molecular ion at m/e 206 and a major fragment with m/e 132 was detected from the compound corresponding to curve B.

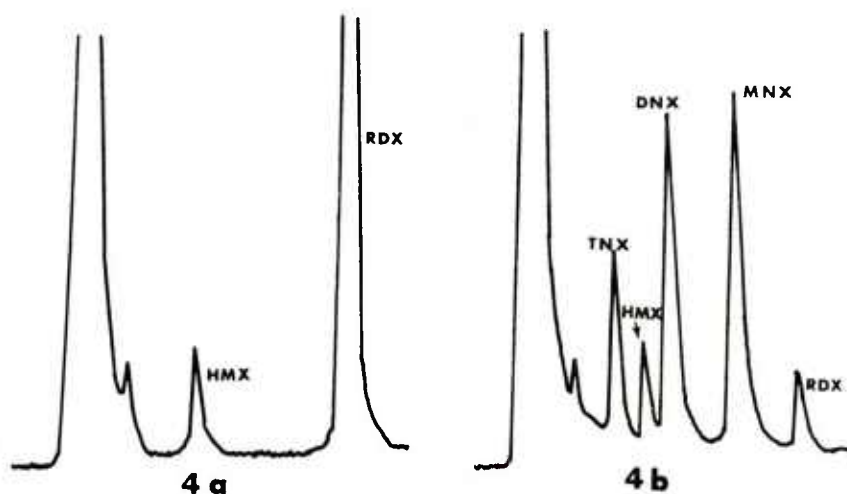


Figure 4. HPLC elution pattern of RDX intermediates.

(Figure 3) and a molecular ion at m/e 190 and fragment ion at m/e 116 from that corresponding to curve C. The material corresponding to curve D was identified as TNX by TLC, HPLC and GC/MS, yielding a molecular ion at m/e 174, and a major fragment ion at m/e 100.

Distribution of Radioactivity from ^{14}C -RDX

A reaction mixture containing 50 mg of RDX/ml including 2 μCi of ^{14}C -RDX, was incubated anaerobically for 7 days. No ^{14}C -labeled gas was evolved during incubation. The incubated mixture was sparged with helium and extracted with ether as described. The results are shown in Table 2. Less than 1.5% of the total ^{14}C added to the system was found in volatile (spargeable) material. Even after three continuous ether extractions approximately 42% of the ^{14}C remained in the aqueous phase. Most of this remaining radioactivity disappeared upon evaporation of the sample to dryness at 45-50°C under a stream of N_2 , regardless of pH. Radioactivity from ^{14}C -labeled RDX was found almost exclusively in the soluble fraction from the earliest measurement (24 hours) to the 21st day, with only 2% associated with the pellet or retained by membrane filters.

Formaldehyde

The presence of HCHO in distillates was demonstrated by the formation of the dimethone derivative and by the characteristic and specific color reaction obtained with chromotropic acid. The amount of HCHO produced from RDX increased to a maximum after 1 to 2 days of incubation (Figure 5) following which the concentration (concn.) declined to a negligible value.

Methanol

The total ^{14}C found in the distillate could not be reconciled with the specific activity expected if all of the ^{14}C in the distillate was present as HCHO . However, initial attempts to identify and quantitate the other ^{14}C -

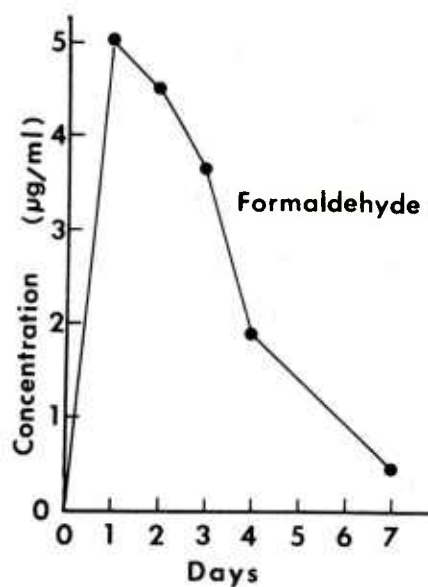


Figure 5. Kinetics of formaldehyde formation.

Table 2. Distribution of radioactivity after anaerobic incubation with ^{14}C -RDX.

Treatment	Radioactivity (%)
None	100.0
Volatiles (from traps)	1.5 ^a
Ether extraction, pH 7 ^b	23.7
Ether extraction, pH 3 ^b	25.4
Ether extraction, pH 11 ^b	7.8
Aqueous phase	41.6

^a Total radioactivity recovered from purging volatiles under acid and alkaline conditions.

^b Continuous ether extraction for 24 hours.

containing material led to the loss of most of the radioactivity. Since the entire reaction was the result of an anaerobic process, there was the possibility that the polar, volatile, low molecular weight, carbon-containing compound might be methanol. Radioactive CMF from reaction mixtures which had been incubated 2 to 3 weeks were subjected to vacuum distillation. Radioactive material collected in a liquid N₂ trap was analyzed by GC/MS. The presence of up to 300 µg of MeOH/ml (9 mM) in the distillate was confirmed. Kinetic data on the formation of MeOH are not yet available.

Hydrazines

The presence of hydrazine, 1,1-dimethylhydrazine and 1,2-dimethylhydrazine was confirmed by GC/MS analysis of the hydrazine derivatives. The bases were isolated as their hydrochloride salts and were then treated with salicylaldehyde or with acetic anhydride to yield either the disalicyl derivative of hydrazine (salicylazine) or the acetyl derivatives of the substituted hydrazines. IR and GC/MS analysis and comparison with authentic salicylazine confirmed the presence of hydrazine in the reaction mixture. Treatment of the salts of the bases with acetic anhydride yielded acetyl derivatives of the dimethylhydrazines. Comparisons with authentic compounds confirmed the presence of both 1,2-dimethylhydrazine and 1,1-dimethylhydrazine.

DISCUSSION

The biodegradation of RDX occurs only under anaerobic conditions. Concurrent with the disappearance of RDX is the sequential build-up and disappearance of the mono-, di-, and trinitroso analogs of RDX (Figure 3). The fact that HCHO formation reaches a maximum in a short time (Figure 5) while that of the nitroso derivatives lags behind, tends to rule out the possibility that HCHO is produced solely by reactions subsequent to the formation of the trinitroso derivative, and suggests that formaldehyde is produced early in the reaction sequence from precursors of trinitroso-RDX. Uninoculated controls containing RDX or TNX and incubated for the same periods of time as the inoculated flasks, produced no HCHO. From the accumulated data we propose a pathway for the biodegradation of RDX as illustrated in Figure 6.

In this scheme RDX is reduced sequentially to the nitroso derivatives, 2 (MNX), 3 (DNX), and 4 (TNX), each of which may undergo further reduction of a nitroso group to form the hypothetical compounds 5 (1-hydroxylamino-3,5-dinitro-1,3,5-triazine), 6 (1-hydroxylamino-3-nitroso-5-nitro-1,3,5-triazine) and 7 (1-hydroxylamino-3,5-dinitroso-1,3,5-triazine). We postulate that the molecule becomes unstable when any one of the nitro groups is reduced beyond the nitroso level. At this point hydrolytic cleavage, followed by rearrangement and further reductions of the fragments, gives rise to the end products observed. Cleavage of 5 via one route yields products 8 (N-hydroxymethyl-methylenedinitramine) and 9 (N-hydroxy-methylenehydrazone), and cleavage of 6 via another route yields 10 (N-hydroxylamino-N'-nitro-methylenediamine) and 11 (dimethylnitrosamine radical). Compound 7 undergoes cleavage via either route.

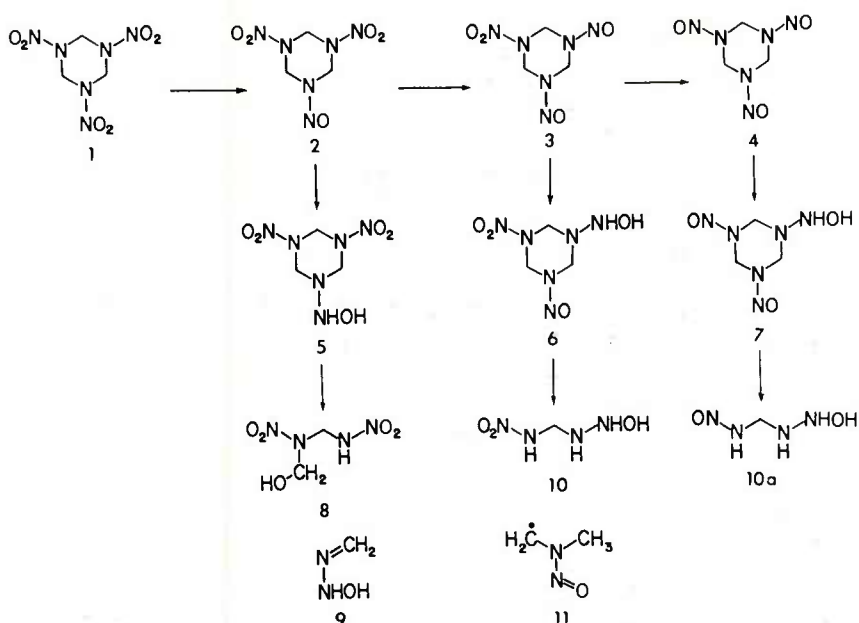


Figure 6. Proposed RDX biodegradation pathway. 1, RDX; 2, MNX; 3, DNX; 4, TNX; 5, 1-hydroxylamino-3,4-dinitro-1,3,5-triazine; 6, 1-hydroxylamino-3-nitroso-5-nitro-1,3,5-triazine; 7, 1-hydroxylamino-3,5-dinitroso-1,3,5-triazine; 8, N-hydroxymethylmethylenedinitramine; 9, N-hydroxymethylenehydrazone; 10, N-hydroxylamino-N'-nitro-methylenediamine; 10a, N-hydroxylamino-N'-nitro-methylenediamine; 11, dimethyl-nitrosamine radical.

Figure 7 shows the postulated reactions of the fragments arising from the initial cleavage reaction. Cleavage of 8 releases 12 (HCHO) and 13 (methylenedinitramine) which decomposes to yield HCHO and 14 (nitramide) which in turn is reduced to 15 (hydrazine). Compound 9 rearranges and is reduced to 16 (hydroxymethylhydrazine) which yields 12 (HCHO) and 15 (hydrazine). Under the strongly reducing conditions HCHO is reduced to 17 (methanol).

The other cleavage reaction (via compounds 6 and 7) yields derivatives of methylenediamine (compounds 10 and 10a) which follow the reductive pathway of compound 13 to hydrazine. As shown in Figure 8, the other product of this cleavage (compound 11) either undergoes sequential reduction to 19 (1,1-dimethylhydrazine) via compound 18 (dimethylnitrosamine) or rearranges via

20 (hypothetical intermediate) to yield 21 (dimethyldiazene-1-oxide radical), which is reduced to 23 (1,2-dimethylhydrazine) via 22 (dimethyldiazene-1-oxide). This scheme allows for the formation of all the observed products.

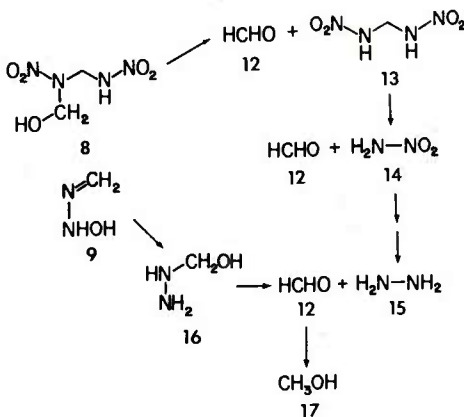


Figure 7. Proposed RDX biodegradation pathway.
12, HCHO; 13, methylenedinitramine;
14, nitramide; 15, hydrazine;
16, hydroxymethylhydrazine;
17, methanol.

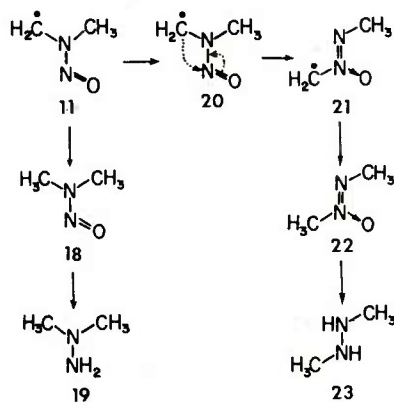


Figure 8. Proposed RDX biodegradation pathway.
18, dimethylnitrosamine;
19, 1,1-dimethylhydrazine;
20, hypothetical intermediate;
21, dimethyldiazene-1-oxide radical;
22, dimethyldiazene-1-oxide;
23, 1,2-dimethylhydrazine.

Figure 5 represents a steady-state concn. reflecting the formation of HCHO and its concomitant reduction to methanol presumably as soon as it is formed, thus the time of maximum net generation of HCHO may not be truly represented. If all the carbon in a solution of 50 μg of RDX/ml (225 μM) was converted to HCHO, a concn. of 676 μM HCHO (20.3 $\mu\text{g}/\text{ml}$) would result. Complete reduction to methanol would yield a methanol concn. of 21.6 $\mu\text{g}/\text{ml}$. The maximum steady-state concn. of HCHO indicated in Figure 5 was 5 $\mu\text{g}/\text{ml}$, so even under steady-state conditions at least 25% of the carbon is accounted for. During the vacuum distillation of 100 ml of CMF_5 , approximately 5 ml of distillate was collected. If 100% efficiency was attained for the quantitative distillation of methanol, then there was a 20-fold concn. achieved. Since as much as 300 μg of methanol/ml were detected in one distillate sample, then the original sample contained 15 μg of methanol/ml, which is consistent with the above. Thus, even in the absence of quantitative kinetic information on the formation of methanol or the hydrazines, an appreciable portion of the carbon can be accounted for. Since some of the carbon must be found in the methyl groups of the dimethylhydrazines, it is likely that we will be able to account for the majority of the carbon.

Several facts emerge pertaining to the nature of the final products remaining in solution: (1) no spargeable, volatile ^{14}C -containing compounds were detected, (2) 98% of the radioactivity remained in the supernatant after the disappearance of RDX, formaldehyde, and the nitroso derivatives of RDX, (3) ^{14}C was ether-extractable from acidic, alkaline, or neutral solutions, and (4) ^{14}C disappeared when either acidic, alkaline, or neutral solutions were evaporated to dryness. These findings strongly suggested the presence of carbon-containing, low molecular weight, polar, neutral compounds. Methanol and formaldehyde subsequently were identified as two compounds with these

properties. The proposed pathway includes several additional compounds that fit this description, namely dimethylnitrosamine and 1,2-dimethyldiazine-1-oxide (azoxymethane) (compounds 18 and 22, respectively), however we have not detected these compounds in reaction mixtures.

The biological treatment of RDX-containing wastes must include an anaerobic mode since no reaction occurs aerobically. Since munitions wastes generally contain high levels of nitrate, RDX wastes can be treated in an anaerobic denitrification mode. The result of such an operation would be a mixture of reduced compounds, including the hydrazines and methanol. Upon subsequent exposure to an aerobic state, methanol would be oxidized to CO₂. Little is known about the biological fate of hydrazine and the dimethylhydrazines under aerobic conditions. Both 1,1- and 1,2-dimethylhydrazine and their immediate precursors, dimethylnitrosamine and azoxymethane, as well as hydrazine, are known mutagens and/or carcinogens^{23,24,25} and therefore it is most important to determine the biodegradability of these compounds. Such studies are currently in progress.

²³Fiala, E. S. 1977. Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. Cancer 40: 2436-2445.

²⁴Greenhouse, G. 1976. Evaluation of the teratogenic effects of hydrazine, methylhydrazine, and dimethylhydrazine on embryos of Xenopus laevis, the South African clawed toad. Teratology 13:167-177.

²⁵Skopek, T. R., et al. 1978. Quantitative forward mutation assay in Salmonella typhimurium using 8-azaguanine resistance as a genetic marker. Proc. Natl. Acad. Sci. (U.S.), 75:410-414.

LITERATURE CITED

- Beasley, R. K., D. E. Hoffman, M. L. Rueppel and J. W. Worley. 1980. Sampling of formaldehyde in air with coated solid sorbent and determination by high performance liquid chromatography. *Anal. Chem.* 52: 1110-1114.
- Blout, E. R. and R. M. Gofstein. 1945. The absorption spectra of certain al-dazines. *J. Am. Chem. Soc.* 67: 13-17.
- Brockman, F. J., D. C. Downing and G. F. Wright. 1949. Nitrolysis of hexamethylenetetramine. III. Preparation of pure cyclonite. *Canad. J. Res.* 27B: 469-474.
- Croce, M. and Y. Okamoto. 1978. Cationic micellar catalysis of the aqueous alkaline hydrolysis of 1,3,5-triaza-1,3,5-trinitrocyclohexane and 1,3,5,7-tetraaza-1,3,5,7-tetranitrocyclooctane. *J. Org. Chem.* 44: 2100-2103.
- Fiala, E. S. 1977. Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. *Cancer.* 40: 2436-2445.
- Glennon, J. P. and L. H. Reuter. 1976. Environmental quality standards for munitions-unique pollutants. Proc. 17th Dept. of Army Explosives Safety Seminar, Denver, CO.
- Glover, D. J. and J. C. Hoffsommer. 1979a. Photolysis of RDX. Identification and reactions of products. Tech. Rep. NSWC TR 79-349. Naval Surf. Weap. Center, Silver Springs, MD.
- Glover, D. J. and J. C. Hoffsommer. 1979b. Photolysis of RDX in aqueous solution, with and without ozone. NSWC/WOL TR 78-175. Naval Surf. Weap. Center, Silver Springs, MD.
- Grant, W. M. 1948. Colorimetric microdetermination of formic acid based on reduction to formaldehyde. *Anal. Chem.* 20: 267-269.
- Greenhouse, G. 1976. Evaluation of the teratogenic effects of hydrazine, methylhydrazine, and dimethylhydrazine on embryos of *Xenopus laevis*, the South African clawed toad. *Teratology.* 13: 167-177.
- Hathaway, J. A. and C. R. Buck. 1977. Absence of health hazards associated with RDX manufacture and use. *J. Occup. Med.* 19: 269-272.
- Hoffsommer, J. C., L. A. Kaplan, D. J. Glover, D. A. Kubose, C. Dickenson, H. Goya, E. G. Kayser, C. L. Groves, and M. E. Sitzmann. 1978. Biodegradability of TNT: A three year pilot study. NSWC/WOL TR 77-136.
- Hoffsommer, J. C., D. A. Kubose and D. J. Glover. 1977. Kinetic isotope effects and intermediate formation for the aqueous alkaline homogeneous hydrolysis of 1,3,5-triaza-1,3,5-trinitrocyclohexane (RDX). *J. Phys. Chem.* 81: 380-385.

LITERATURE CITED (cont'd)

- Jackson, R. A., J. M. Green, R. L. Hash, D. C. Lindsten and A. F. Tatyrek. 1978. Nitramine (RDX-HMX) wastewater treatment at the Holston Army Ammunition Plant. Report ARLCD-CR-77013, U.S. Army Armament Res. and Dev. Command, Dover, NJ.
- Osmon, J. and R. E. Klausmeier. 1973. Microbial degradation of explosives. Dev. Ind. Microbiol. 14: 247-252.
- Ross, E. R. 1976. Two-year chronic toxicity study in rats. US NTIS, AD Rep. AD-A04061.
- Schiessler, R. W. and J. H. Ross. 1948. U.S. Patent 2,434,230.
- Schneider, N. R., S. L. Bradley and M. E. Andersen. 1977. Toxicology of cyclotrimethylenetrinitramine (RDX): distribution and metabolism in the rat and the miniature swine. Toxicol. Appl. Pharmacol. 39: 531-541.
- Shriner, R. L. and R. C. Fuson. 1948. The systematic identification of organic compounds. John Wiley and Sons, Inc., New York, NY.
- Sikka, H. C., S. Banerjee, E. J. Pack and H. T. Appleton. 1980. Environmental fate of RDX and TNT. Report DAMD 17-77-C-7026. U.S. Army Med. Res. & Dev. Command, Fort Detrick, Frederick, MD.
- Simacek, J. 1957. Decomposition of nitrosamines and nitramines in protogenic solvents. III. Synthesis of N,N'-dinitro-N"-nitrosocyclotrimethylenetriamine. Chem. listy. 51: 2367-2368.
- Skopek, T. R., H. L. Liber, J. J. Krolewski and W. G. Thilly. Quantitative forward mutation assay in Salmonella typhimurium using 8-azaguanine resistance as a genetic marker. Proc. Natl. Acad. Sci. (U.S.) 75: 410-414.
- Soli, G. 1973. Microbial degradation of cyclonite (RDX). US Nat. Tech. Inform. Serv. AD Rep. No. 762751.
- Sullivan, J. H., H. D. Putnam, A. A. Keirn, J. C. Nichols and J. T. McClave. 1979. A summary and evaluation of aquatic environmental data in relation to establishing water quality criteria for munitions unique compounds. Part 4: RDX and HMX. Report DAMD 17-77-C-7027. U.S. Army Med. Res. & Dev. Command, Fort Detrick, Frederick, MD.
- Walker, J. F. 1944. Formaldehyde. Reinhold Publishing Corp., New York, NY.